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RESEARCH PAPER

Transport of sucrose, not hexose, in the phloem

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Abstract

Several lines of evidence indicate that glucose and fructose are essentially absent in mobile phloem sap. However, this paradigm has been called into question, especially but not entirely, with respect to species in the Ranunculaceae and Papaveraceae. In the experiments in question, phloem sap was obtained by detaching leaves and placing the cut ends of the petioles in an EDTA solution. More hexose than sucrose was detected. In the present study, these results were confirmed for four species. However, almost identical results were obtained when the leaf blades were removed and only petiole stubs were immersed. This suggests that the sugars in the EDTA solution represent compounds extracted from the petioles, rather than sugars in transit in the phloem. In further experiments, the leaf blades were exposed to $^{14}CO_2$ and, following a chase period, radiolabelled sugars in the petioles and EDTA exudate were identified. Almost all the radiolabel was in the form of [^{14}C]sucrose, with little radiolabelled hexose. The data support the long-held contention that sucrose is a ubiquitous transport sugar, but hexoses are essentially absent in the phloem stream.

Key words: EDTA, fructose, glucose, hexose, phloem, sucrose.

Introduction

There has been a consensus in the phloem transport field for over 50 years that sucrose is the predominant sugar carried in the sieve tubes of most species. By contrast, hexoses are considered to be non-mobile. These concepts derived from early chemical analyses of dissected phloem and phloem exudate, but were put on firmer experimental grounds when ^{14}C became available as a tracer. In perhaps the first compelling study of this type, Swanson and El-Shishiny (1958) exposed grape leaves to $^{14}CO_2$. Hours later they detected [^{14}C]sucrose, accompanied by small amounts of [^{14}C]hexose, in the bark near the transport front. They concluded that sucrose is the transported sugar, whereas the small amount of radiolabelled hexose had been derived from [^{14}C]sucrose by hydrolysis. Subsequent $^{14}CO_2$ studies in other plants have similarly found large amounts of radiolabelled sucrose, often with small amounts of accompanying radiolabelled hexoses (Geiger and Swanson, 1965; Mortimer, 1965).

Since that time, the virtual absence of hexoses in the phloem has been confirmed by other techniques, such as sampling phloem by bark incisions (Zimmermann and Ziegler, 1975) and aphid stylectomy (Fisher and Frame, 1984). The fluid obtained from severed aphid stylets is usually considered to be the most authentic source of unadulterated sieve tube sap and, to the best of our knowledge, no stylectomy study to date has found more than trace amounts of hexose in the collected fluid (Fukumorita and Chino, 1982; Fisher and Frame, 1984; Weibull *et al.*, 1990; van Helden *et al.*, 1994; Amiard *et al.*, 2004).

However, in a recent study, van Bel and Hess (2008) concluded that, in many species, especially those in the Ranunculaceae and Papaveraceae, but in other families as well, a large percentage of the carbohydrate in the phloem exudate is in the form of hexose. Indeed, in some species the amount of hexose in the exudate was up to 21-fold in excess of sucrose. In other plants a much lower hexose-to-sucrose ratio was found. These experiments were conducted using the facilitated-exudation technique introduced by King and Zeevaart (1974), in which ethylenediaminetetraacetic acid (EDTA) is used to prevent the sieve tubes of severed vascular bundles from sealing.

In the original experiments, King and Zeevaart (1974) exposed blades of attached leaves to $^{14}CO_2$, then cut the petioles and placed the cut ends in collection solution. In the absence of EDTA there was little or no exudation of ^{14}C, but in the presence of EDTA, radiolabel exuded for many hours. Since the analyses focused specifically on radiolabelled compounds, it was clear that these substances had been exported from the leaf blade and were in transit down the petiole in the phloem. ^{14}C-labelled hexoses were not detected in the exudate from the species studied, namely *Perilla crispa*, *Chenopodium rubrum*, and *Pharbitis nil*.

In the study by van Bel and Hess (2008), the leaves of the plants in question were not radiolabelled, a departure from the original procedure that is commonly made in EDTA-exudation studies. Thus, there is the possibility that the compounds in the collection solution represent not only those in transit in the sieve tubes, but also contaminants from other tissues. There is a danger of contamination since EDTA chelates Ca^{2+} in membranes and cell walls, softening tissue, and inducing leakage of electrolytes and metabolites, including carbohydrates (Foote and Hanson, 1964; van Steveninck, 1965; Aloni *et al.*, 1986; Hepler, 2005).

Experiments were conducted on three species studied by van Bel and Hess (2008) with especially high hexose:sucrose ratios, plus an additional species. Leaf blades were exposed to $^{14}CO_2$ and the radiolabelled compounds transported to the petioles and into EDTA solution were analysed. Radiolabel was found primarily in sucrose, with only small amounts of radiolabelled hexose. Unlabelled sugars were also analysed in EDTA-exudation studies on the same species, comparing whole leaves with stubs of petioles alone. The results indicate that, essentially, all of the exuded sugars are extracted from the petioles and do not represent mobile sugars in the phloem.

Materials and methods

Plant materials

Anemone sylvestris, *Centranthus ruber*, *Digitalis purpurea*, and *Pulsatilla vulgaris* plants were grown in Metro Mix 360 (Scotts, Marysville, Ohio) under ambient conditions in a greenhouse. *A. sylvestris* was substituted for *Anemone nemorosa*, used by van Bel and Hess (2008) because the latter was not available to us. EDTA experiments were conducted during the summer and ^{14}C experiments at several times throughout the year.

EDTA-facilitated exudation

Mature leaves were excised by cutting the petioles 4–7 cm from the leaf blade under the surface of 5 mM 2-Na-ethylenediaminetetra-acetic acid, disodium salt (EDTA), pH 7.0. Fifteen minutes later, the petioles of 3–5 excised leaves, depending on size, were inserted into 1.5 ml Eppendorf tubes with 1.0 ml of EDTA solution. Due to their size, 50 ml tubes were used for *D. purpurea* leaves. Leaf blades were cut from the petioles in half of the tubes on a random basis. Tubes were then placed in a closed chamber under low light conditions (<150 μmol photons m^{-2} s^{-1}) and close to 100% humidity to reduce transpiration. High humidity was achieved by placing wet paper towels on the inner surface of the chamber with water at the bottom. Every hour, over a 6 h period, samples were transferred into fresh medium in new tubes, while the old tubes were immediately frozen in liquid nitrogen and stored in a freezer at –20 °C. The first 1 h tube was discarded and the remaining hourly samples were combined.

Polyclar (0.025 g ml^{-1}; Sigma, St Louis, MO) was added to the samples to remove phenolic compounds, the tubes were placed in boiling water for 10 min to stop enzymatic activity, and the cooled samples were centrifuged at 1000 rpm for 10 min. Residual Polyclar was removed from 0.6 ml samples with a Corning Spin-X Centrifuge Filter. The supernatant was passed through a 3-layer ion-exchange column consisting of 1.2 ml AG 50W-X4 (hydrogen form), 0.6 ml PVPP (polyvinylpolypyrrolidone) and 1.2 ml Amberlight DRA-67 resins. AG50W-X4 resin was obtained from Bio-Rad, Hercules, CA. PVPP and Amberlite IRA-67 were obtained from Sigma, St Louis, MO. Sugars were analysed by high performance liquid Chromatography (HPLC) using a Waters 510 HPLC pump, Waters 2414 Refractive Index Detector, Agilent 1200 Series Autosampler, and Waters Sugar-Pak 6.5×300 mm HPLC column.

Radiolabelling

Plants were freshly watered and brought to a laboratory fume-hood equipped with a water-filtered incandescent 1000-W metal-halide lamp (600 μmol photons m^{-2} s^{-1}) for approximately 1 h prior to labelling. Mature, attached leaves were enclosed in a gas-tight cuvette, into which $^{14}CO_2$ (1.0 MBq) was introduced with a syringe. The $^{14}CO_2$ was generated by the addition of excess 80% (v/v) lactic acid to $Na_2^{14}CO_3$ (6.6 10^5 MBq $mmol^{-1}$). After 15–30 min of exposure to the $^{14}CO_2$, the leaves were removed from the cuvette and exposed to room air during the chase period. During the labelling and chase periods the petioles were wrapped in aluminium foil to prevent fixation of radiolabel from inadvertent leakage of $^{14}CO_2$.

Chase periods were experimentally determined to maximize radioactive content in the petioles. *C. ruber* was chased for 1 h, *A. sylvestris* and *D. purpurea* for 2 h, and *P. vulgaris* for 6 h. Following the chase period, the leaf blade and the petiole were excised from the plant, separately frozen in liquid N_2 and crushed finely in a mortar. Tissue was extracted with methanol, chloroform, and water (12:5:3 by vol.) and the aqueous phase, separated by the addition of water (0.6 vols), was purified through anion and cation exchange columns, as described above. The neutral phase was analysed by thin layer chromatography (TLC) on Whatman TLC plates (250 μm, Silica Gel 60 A) with acetic acid, chloroform, and water (7:6:1 by vol.) as solvent system, run twice to achieve maximum separation. Radiolabelled spots were localized with X-ray film (Kodak BioMax MR film, Rochester, NY) and spots scraped from the plates were counted in Ecoscint scintillation solution (National Diagnostics, Atlanta, GA, USA).

To obtain ^{14}C-labelled exudate, attached leaves were exposed to $^{14}CO_2$ for 30 min, as described above. The leaves were then cut under water and the cut ends of the petioles were immersed in tubes of water for 15 min under dim light, until transpiration ceased. The ends of the petioles were then trimmed again under EDTA and the petioles immersed in tubes containing 20 mM EDTA, pH 7.0. Exudate was collected for 4 h beginning 2 h after labelling was begun. Samples were passed through ion exchange columns and analysed by TLC and scintillation counting, as described above.

Results

$^{14}CO_2$ pulse–chase experiments on attached leaves

To identify sugars that are mobile in the sieve tubes, blades of mature, attached leaves were exposed to $^{14}CO_2$ and radiolabelled transport compounds were given time during

a chase period to migrate into the petioles (see Materials and methods). During the labelling period the petioles were wrapped in aluminium foil to prevent inadvertent exposure to $^{14}CO_2$. Following the chase period, leaf blades and petioles were separately extracted, and the radiolabelled sugars were identified by TLC and scintillation counting.

In leaf blades, sucrose was the predominant radiolabelled sugar, with smaller but nonetheless significant amounts of radiolabelled hexose. Radiolabelled hexose constituted from 13% (*C. ruber*) to 47% (*D. purpurea*) of radiolabelled sugar (Fig. 1A) in the blades. In the petioles, almost all the radiolabelled sugar was sucrose (Fig. 1B), with much lesser amounts of hexose, ranging from 5% of total radiolabelled sugar in *C. ruber* to 14% in *D. purpurea*. In all species, the proportion of radiolabelled sucrose to hexose was higher in the petiole than in the leaf blade.

EDTA exudation experiments

EDTA-facilitated exudation experiments were conducted as described by van Bel and Hess (2008) and in the Materials and methods. In preliminary experiments, the petioles of excised leaves were placed in vials containing different concentrations of EDTA solution, from 2.5–20 mM, and the EDTA solution was collected and changed at hourly intervals for 6 h. As reported by van Bel and Hess (2008), the amount of sugar exuded varied at different EDTA concentrations, and at different time intervals, but the ratio of sucrose to hexose exuded did not vary. Therefore, subsequent studies were conducted using 5 mM EDTA. The EDTA solution was collected at hourly intervals. The first 1 h sample was discarded to avoid contamination from cut cells, and since the proportions of the various sugars remained the same over the next 5 h (see Supplementary Fig. S1 at *JXB* online), the remaining five hourly samples were combined for HPLC analysis.

The results were more variable than those from the $^{14}CO_2$ pulse–chase experiments, both in the total amount and in the proportions of the various sugars exuded (Fig. 2; whole-leaf). Sucrose, glucose, and fructose were detected in all experiments and the proportion of hexose was considerably higher than in the radiolabelling studies described above. Indeed, in all species except *C. ruber*, the combined quantity of glucose and fructose exceeded that of sucrose.

To determine if the sugar in the EDTA solutions at the end of the exudation period came from the leaf blades, control experiments were concurrently conducted in which the leaf blades were removed, with only the remaining stubs of petioles in the EDTA solution (Fig. 2; petiole). Care was taken to prevent the EDTA from coming into contact with the upper cut surfaces of the petiole stubs to limit extraction to only one cut surface. In all species, the proportions of the exuded sugars from the petioles were similar to those exuded from whole leaves. In *C. ruber*, *D. purpurea*, and *P. vulgaris*, there was no statistically significant difference in the total sugar exuded in samples of whole leaves and petiole stubs. In *A. sylvestris*, there was consistently more sugar in the samples of exudate from petiole stubs than in those collected from whole leaves.

To determine if invertase activity was responsible for the presence of hexose in the exudation fluid, experiments were conducted with *D. purpurea* in which [^{14}C]sucrose was added to the exudation solution at the beginning of the 6 h experiment. At the end of the experiment, 3.9% (±2.3%; SE, n=3) of the [^{14}C]sucrose had been hydrolysed to [^{14}C]hexose.

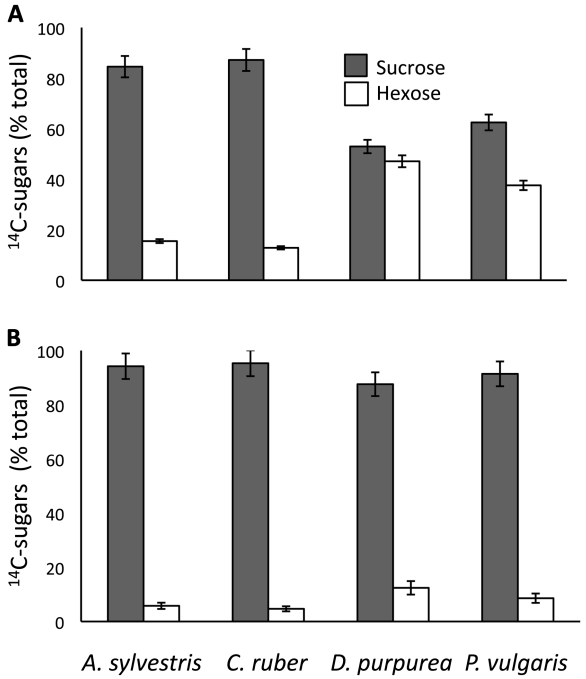

Fig. 1. Radiolabelled sugars in $^{14}CO_2$ pulse-chase experiments. Attached leaf blades were exposed to $^{14}CO_2$. Following a chase period, leaf blades (A) and petioles (B) were separately extracted and analysed. Error bars represent standard errors (n=3).

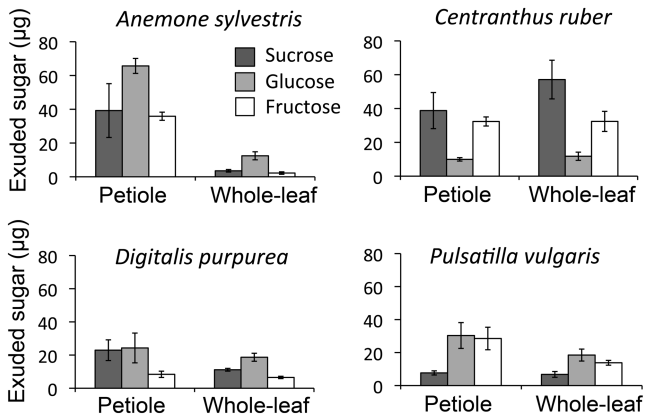

Fig. 2. EDTA-mediated exudation from petioles and whole-leaves. The cut ends of petioles of whole leaves, or the petioles alone, were submerged in a tube containing 5 mM EDTA solution. Three petioles or whole-leaves were used per tube for *D. purpurea* and five petioles or whole-leaves for each of the other three species. The solutions were replaced every hour for 6 h. The 1 h sample was discarded and the remaining samples were pooled. Sugars were quantified by HPLC. Error bars represent standard errors (n=3).

To analyse ^{14}C-labelled sugars exuded into collection fluid, leaf blades of intact *D. purpurea* plants were provided with $^{14}CO_2$ and the leaves were then detached and the cut petioles were immersed in EDTA solution. The radiolabelled carbohydrates exuded from the petioles over the next 6 h consisted of 77.7% sucrose (±1.2%; SE, n=3), 14.6% sugar alcohol (±0.3%; SE, n=3), and 7.7% hexose (±1.4%; SE, n=3).

Discussion

The experiments reported here indicate that sucrose is the dominant, and perhaps the only, sugar transported in the phloem of *A. sylvestris*, *C. ruber*, *D. purpurea*, and *P. vulgaris*. These plants have been reported to transport more hexose than sucrose (van Bel and Hess, 2008). When the leaf blades were exposed to $^{14}CO_2$, radiolabelled sucrose, accompanied by a small amount of radiolabelled hexose, was subsequently found in the petioles of attached leaves.

The presence of small quantities of [^{14}C]hexose in the petioles is probably due to metabolism of [^{14}C]sucrose in the phloem and surrounding cells. Radiolabelled hexoses and other metabolites such as amino acids are commonly found along the translocation route in studies where assimilates are photosynthetically labelled with $^{14}CO_2$ (Webb and Gorham, 1964; Geiger *et al.*, 1969; Fisher, 1970), yet hexoses are not found in sap exuding from cut aphid stylets (Fukumorita and Chino, 1982; Fisher and Frame, 1984; Weibull *et al.*, 1990; van Helden *et al.*, 1994; Amiard *et al.*, 2004). In this respect, the radiolabelling data from the four species examined here are similar to those from other plants that have been analysed by the same $^{14}CO_2$ technique. Therefore, there is no reason to suggest that the composition of the mobile sugars in these plants is unique or even unusual.

As a further test, exudation experiments were conducted essentially as described by King and Zeevaart (1974), labelling *D. purpurea* leaf blades with $^{14}CO_2$ and analysing radiolabelled sugars exuded into the EDTA solution. Again, almost all the radiolabel was found in sucrose.

If sucrose is the dominant, and perhaps exclusive, mobile sugar in the phloem, why are unlabelled hexoses so prevalent in the EDTA-facilitated exudation studies? One possibility is that sucrose effluxes into the collection solution, but is then hydrolysed to glucose and fructose by invertase. van Bel and Hess (2008) tested this possibility by removing leaves from collection tubes and measuring the subsequent hydrolysis of sucrose over time. Invertase activity was noted, but it was not of sufficient magnitude to explain the high hexose levels. Relatively low invertase activities in EDTA-facilitated exudate were also reported by Groussol *et al.* (1986). Our data demonstrating relatively little hydrolysis of [^{14}C]sucrose added to the exudation solution confirm these results. Therefore, invertase activity alone cannot explain the presence of excessive concentrations of hexose in the collection fluid. However, invertase activity can explain the presence of small amounts of ^{14}C-hexose in exudate when leaf blades are exposed to $^{14}CO_2$. When leaf blades of *D. purpurea* were exposed to $^{14}CO_2$, 7.7% of the radiolabel in the EDTA exudate was in the form of hexose. When pure sucrose was added directly to the EDTA solution during the exudation period 3.9% was hydrolysed to hexose. Therefore, much of the exuded ^{14}C-hexose can be accounted for as a hydrolysis product; little, if any, ^{14}C-hexose is exuded as a mobile constituent of the phloem sap.

In cases where there is abundant unlabelled hexose in the collection fluid, the most likely explanation is that EDTA compromises the cell integrity of the immersed tissue by chelating Ca^{2+}, leading to a leakage of cell contents (Foote and Hanson, 1964; Van Steveninck, 1965; Aloni *et al.*, 1986; Hepler, 2005). van Bel and Hess (2008) dismissed this possibility on the basis that the efflux rate from petiole stubs, in their experiments, did not increase over time, as would be expected if EDTA had a deleterious effect on cells. However, an increase in the efflux rate due to progressive damage by the EDTA solution is an untested assumption. A more direct method of measuring the effect of EDTA is to compare the efflux from petiole stubs and whole leaves. In our experiments, there was as much sugar in collection fluid from petiole stubs, and in approximately the same proportions of sucrose and hexoses, as from whole leaves. A reasonable conclusion is that the EDTA extracts so much sugar, both hexose and sucrose, from the petiole that it overwhelms and masks any sugar exuding from the phloem. It is reasonable to assume that some sugar exudes from the phloem, although the experiments in which radiolabel in the EDTA solution was analysed when leaves of *D. purpurea* were exposed to $^{14}CO_2$ suggest that the ratio of exuded sugars differs substantially from that of the EDTA solution in general.

Interestingly, in our experiments with *A. sylvestris*, the amount of carbohydrate in the collection solution was consistently higher with petiole stubs than with whole leaves. This is attributed to the uptake of solution into whole leaves by transpiration, leading to sample volume loss, as observed by van Bel and Hess (2008) and Araki *et al.* (1997).

Why are hexoses not transported in the phloem? According to Arnold (1968), sucrose and other non-reducing sugars and sugar alcohols have been evolutionarily selected as transport compounds because they are less reactive than reducing sugars. The carbonyl groups of sugar molecules form covalent adducts with protein amino groups by the Maillard reaction (Yamauchi *et al.*, 2002). Indeed, Bechtold *et al.* (2009) consider the glucose-induced glycation threat to be on a similar scale to that of reactive oxygen and nitrogen species. Note that when hexoses were generated in companion cells of transgenic potato by yeast invertase, at least some of the hexose was converted to the non-reducing trisaccharide 6-kestose, perhaps as a defence against this chemical hazard (Zuther *et al.*, 2004). Considering the very high concentrations of sugars maintained in the phloem, the consequences of hexose transport would appear to be especially detrimental to all components of the transport system: the sieve tubes, companion cells, and receiving sink cells.

While these teleological arguments are reasonable, they do not explain the absence of hexoses in the phloem in a mechanistic sense. Kallarackal and Komor (1989)

demonstrated that glucose and fructose are transported in the phloem of *Ricinus communis* if the cotyledons are incubated in these sugars. Therefore, the chemical nature of the monosaccharides is not incompatible with long-distance transport. The mechanistic reason that they are not transported out of leaves under normal circumstances appears to be that they do not have access to the phloem. Almost all hexose molecules are sequestered in vacuoles. In four species studied by the non-aqueous fractionation technique, 96–98% of the glucose and fructose in leaf blades was confined to the vacuolar fraction (Nadwodnik and Lohaus, 2008). Essentially the same result was obtained for tobacco (Heineke *et al.*, 1994). If hexoses reside in the vacuoles, they cannot enter the phloem. The absence of hexose in aphid stylet exudate suggests that any hexose molecules that do enter the sieve tubes, or are generated in companion cells by sucrose hydrolysis, are metabolized.

In summary, experiments in which carbohydrates are radiolabelled by photosynthetic incorporation of ^{14}C identified sucrose as the primary, if not the only, mobile sugar in the phloem of the four species examined in this study. By contrast, hexoses were the most abundant unlabelled sugars obtained by submerging the cut ends of petioles in an EDTA solution. Most, if not all, of the hexose in the EDTA solution was derived, not from the phloem, but from the petiolar tissue itself.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Time-course of EDTA-mediated exudation from petioles and whole leaves of *Centranthus ruber*, as analysed by HPLC: the relative proportions of sucrose, glucose, and fructose are shown.

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